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Genetics of *sanguinis*-group streptococci in health and disease

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1 INTRODUCTION

2 Our view of oral streptococci has largely been influenced by the approach taken in
3 the last century to identify etiologic agents of disease. As a consequence, beneficial
4 aspects of streptococcal colonization of the oral cavity were initially overlooked. The first
5 comprehensive analysis of the resident oral microbiota was accomplished in 2005 (1) and
6 with this, a new picture began to emerge. With the availability of high throughput
7 sequencing techniques and an increased sensitivity in analysis methods, the presence of
8 a defined microbiome associated with oral health has been shown (2). Alongside this,
9 'omics' techniques have revealed that prevalent oral diseases such as caries and
10 periodontal disease are polymicrobial in nature and the result of microbial dysbiosis (3, 4).
11 Even more striking, the metabolic output of these mixed microbial communities seems to
12 be more relevant than their precise microbial composition (4). This is also reflected by the
13 fact that the severity of caries and periodontal disease is heavily influenced by the
14 synergistic interactions of the individual members of the polymicrobial consortium,
15 including metabolic cross-feeding and interspecies signaling with transcriptional
16 adjustment to the metabolic output. Thus, the ecological context of the microbial
17 community seems to be of importance to understand oral health and disease
18 development. As a consequence, polymicrobial diseases cannot be explained by the
19 behavior of one bacterial species and certainly cannot be treated like diseases that follow
20 Koch's postulates (5-7). Novel approaches to combat oral polymicrobial diseases should
21 therefore focus on the bacterial community that is present in the healthy oral cavity. Since
22 oral streptococci are abundant during initial colonization of the tooth (8, 9), their function
23 is to provide a favorable environment for incorporation of later species and to support

accretion of the mature oral biofilm, which in general has a health-protecting function (10, 11).

One of the oral *Streptococcus* species that is repeatedly isolated in great abundance as part of the health-associated microbiome is the initial colonizer *Streptococcus sanguinis* (12-14). Because of the strong association of *S. sanguinis* with oral health, this commensal can serve as a model to understand how an individual species is able to interact with other members of the bacterial community to shape the composition of a benign oral biofilm. *S. sanguinis* is in general classified as a non-spore-forming, catalase-negative, chain-forming coccus. *S. sanguinis* is non-beta-hemolytic, but is able to produce a green coloration on blood agar plates referred to as *alpha*-hemolysis, which is a consequence of hydrogen peroxide (H₂O₂) production (15). *S. sanguinis* has been placed into the *mitis* group of streptococci based on 16S rRNA sequence analysis; however, it has also been classified in its own group together with *S. gordonii* and *S. parasanguinis* (15). A more recent analysis using the housekeeping genes *rpoB*, *sodA*, *ddl*, and *gdh* showed a more distant relationship with *S. parasanguinis*, but confirmed the phylogenetic relationship with *S. gordonii* (16). Here we present the *sanguinis*-group streptococci in the context of molecular commensalism, highlighting those aspects of their biology that are important for health-associated biofilm development, including polymicrobial interactions, regulatory and mechanistic events (Figure 1).

MOLECULAR DETERMINANTS OF *S. SANGUINIS* AS A COMMENSAL PIONEER COLONIZER

Initial colonization is intimately linked to the adhesion capabilities of oral streptococci. *S. sanguinis*, together with *S. gordonii*, *S. oralis* and *S. mitis*, are well

48 adapted for initial colonization. These streptococci express a multitude of bacterial surface
49 proteins, named adhesins, which are able to interact with salivary proteins covering the
50 tooth surface (17-19). *S. sanguinis* seems to be particularly well equipped with adhesins
51 that recognize this salivary pellicle (20). Overall, initial colonization has two important
52 consequences. First, the ecological niche is occupied, which plays a significant role in the
53 process of colonization resistance and the exclusion of potential incoming pathogens.
54 Second, initial colonization shapes the overall composition of the biofilm, since the pioneer
55 colonizer can produce metabolic products or provide a substratum for compatible partner
56 species. Initial biofilm development requires the formation of macromolecular complexes.
57 Complex formation is facilitated through electrostatic interactions of salivary proteins with
58 the tooth surface to form the acquired enamel pellicle, a process that occurs within
59 seconds after a clean enamel surface is exposed to saliva (21). Microbial attachment to
60 the acquired enamel pellicle is then mediated via protein-protein and lectin-like
61 interactions. The protein content of the acquired enamel pellicle is thus a major
62 determinant of colonization sequence, dictating strength of microbial adhesion as well as
63 localization, since pellicle proteins seem to differ according to anatomical site (22).

64 A prominent protein in saliva and the acquired enamel pellicle is α -amylase,
65 responsible for the catalytic hydrolysis of starch (23, 24). Amylase binding proteins have
66 been identified in several oral streptococcal species (24). Best studied is the amylase-
67 binding protein A (AbpA) in *S. gordonii* (25). Mutation of AbpA results in deficient biofilm
68 formation and bacterial adhesion *in vitro* (26). Although the sequenced reference strain *S.*
69 *sanguinis* SK36 seems to encode an *abpA* homolog in a similar chromosomal context with
70 its accessory sortase, *srtB* (27), its function is currently unknown. Interestingly, *S.*

sanguinis is able to bind directly to surface-bound amylase and *vice versa* (24). This function has been shown to be mediated by long filamentous pili, organized in a four-gene operon (ssa1631-1634) that encodes three pilin subunits (PilA-C) and a dedicated sortase, SrtC, for cell-surface anchoring of the pilin structural proteins (28). Pili are relatively long and thin appendages and the pili of *S. sanguinis* can be as long as 1 μm , as shown by immune-gold staining of PilA (28). This poses a potentially interesting dynamic with AbpA, which is strictly confined to the outer cell surface, as shown for *S. parasanguinis*, also with immune-gold labeling (27). Taking into account that pili are flexible, one could hypothesize that binding to amylase in acquired enamel pellicle is possible even when the molecule is scarce, since the pili could serve as a flexible “arm”, latching onto free amylase within saliva. A recent publication demonstrating that amylase in acquired enamel pellicle is actually less abundant when compared to saliva (29) is in agreement with this hypothesis, suggesting an advantage of a flexible “arm” over a rigid arrangement on the bacterial surface. The pili also showed binding to other salivary proteins and their deletion diminished biofilm formation on saliva-coated surfaces (28). However, the mutant was still able to bind amylase, albeit with lower efficiency (28), suggesting that other surface proteins are also able to bind amylase, possibly the aforementioned AbpA homolog (27). A pilus-bound amylase also offers the advantage of retaining about 50% of its enzymatic function (30). Gaining access to the amylase substrate starch through a flexible pilus would increase the chance for hydrolysis of the alpha-1,4-glycosidic linkage into glucose, maltose and maltodextrins, promoting sugar uptake and subsequent metabolism by simply increasing the accessible radius of the cell.

Two major mucins are found in saliva, MUC7 (low molecular weight) and MUC5B (high molecular weight) (31, 32). The majority of mucins are synthesized and secreted

95 by the submandibular and sublingual glands, as well as minor glands located in the
96 palatal, buccal and labial mucosae. Mucins are heavily glycosylated glycoproteins and
97 form a lubricating, viscoelastic coating on all oral surfaces. They are abundant proteins
98 in saliva and the acquired enamel pellicle (31, 32). Both MUC7 and MUC5B contain
99 sialic acid as a glycoconjugate and this can be targeted by *S. sanguinis* SK36 sialic-
100 acid-binding adhesin SrpA (33). SrpA contains a subdomain in its binding region that is
101 similar to the V-set Ig-like fold adopted by mammalian Siglecs (sialic acid-binding
102 immunoglobulin-like lectins) (34, 35). Indeed, Siglec-like domains have been identified
103 in potential adhesins of several *S. sanguinis* isolates and other oral streptococci (34).
104 Glycoarray dot blots with human salivary samples and naturally occurring
105 glycoconjugates have demonstrated a high specificity of *S. sanguinis* SK36 SrpA for
106 MUC7, but no binding to MUC5B. This is in contrast to *S. gordonii*, which showed in
107 general better binding to several glycoconjugates, including MUC5B and amylase (34),
108 which are known to form a heterotypic complex (36). As mentioned above, amylase and
109 MUC5B seem to be depleted in the acquired enamel pellicle compared to saliva (29),
110 but this was not seen for MUC7. Thus specificity of binding to MUC7 within the acquired
111 enamel pellicle may go some way to explain why *S. sanguinis* seems to be one of the
112 first oral colonizers and found in greater abundance compared to *S. gordonii*. The
113 glycoarray also revealed an interesting role for divalent cations Ca^{2+} and Mg^{2+} in the
114 binding of *S. sanguinis* SK36 to MUC7, as well as to other glycoconjugates (34).
115 Chelation of Ca^{2+} and Mg^{2+} decreased binding to several of the tested components.
116 This was also observed with two *S. gordonii* strains (34), suggesting a dominant role
117 for divalent cations in the binding process of not only *S. sanguinis*, but of other oral
118 streptococci to salivary components and the acquired enamel pellicle. This is further

supported by the increased abundance of proteins known to bind divalent cations like Ca^{2+} in the acquired enamel pellicle compared to saliva (29). Interestingly, *S. sanguinis* encodes a surface-associated, dual-function protein that bridges substratum attachment and interactions with divalent cations. SsaB is able to bind to saliva-coated hydroxyapatite through an unknown mechanism (37), but its principal function seems to be the transport of divalent cations (38). While this transport was demonstrated to be specific for Mn^{2+} and Fe^{2+} , playing a pivotal role in oxidative stress defense (38), other divalent cations like Ca^{2+} and Mg^{2+} might still be able to interact with SsaB to facilitate binding to the acquired enamel pellicle.

Recently the ability of *S. sanguinis* to be motile on surfaces like solidified agar has been reported for several strains. Spreading zones after prolonged incubation can be observed around colonies, indicating active movement (39). Whether or not this so-called 'twitching motility' has any function or is used as a means to disseminate within the oral biofilm is not yet clear. However, the *pil* locus that encodes the type IV pilus involved in twitching motility is conserved in most of the sequenced *S. sanguinis* genomes (39), suggesting biological importance.

Overall, *S. sanguinis* does not rely on a single mechanism to bind and establish itself within the acquired enamel pellicle. Rather the process is elaborate and ensures the role of *S. sanguinis* as a pioneer colonizer. This correlates with the observation that signal peptidase I is required for biofilm development (40). In general, signal peptidases are membrane-bound endo-proteases that cleave the signal peptide portion from the majority of secreted proteins (41). *S. sanguinis* encodes two signal peptidases, SSA_0849 and SSA_0351, which are crucial for biofilm formation. Deletion of SSA_0351 abolishes biofilm formation but does not affect planktonic growth. Although

the substrates for signal peptidase processing are not experimentally verified, *in silico* prediction identified 168 potential candidates, including several adhesins (40). Further characterization of signal peptidase processed surface proteins will most likely identify new proteins involved in the adhesion process.

S. SANGUINIS IN BIOFILM FORMATION

The initial step in biofilm development is attachment, followed by micro-colony formation of newly attached cells through growth expansion. This process then leads into a series of ordered and temporal events, in which coadhesion predominates, ultimately resulting in formation of a mature biofilm. The production of extracellular matrix material is a defining step in biofilm development and maturation. Matrix materials include polymers such as carbohydrates, proteins, extracellular DNA (eDNA) and lipids, and are collectively referred to as extracellular polymeric substances (EPS) (42). Since EPS is produced by the biofilm inhabitants themselves, specific enzymatic functions and cellular processes are associated with its formation.

Exploiting the intake of dietary sugars, many oral *Streptococcus* species have evolved glucosyltransferases (Gtfs) that hydrolyze sucrose and polymerize the glucose into glucans. These, in turn, promote biofilm development. A single Gtf, GtfP, is carried on the *S. sanguinis* genome, which synthesizes mainly water-soluble α -1,6-linked glucans that branch at α -3,6-linked glucose residues (43). TetR family regulator BrpT was recently identified as a repressor of *gtfP* expression. While a *gtfP* mutant formed only a fragile biofilm, the biofilm formed by the *brpT* mutant was thicker, more robust, and with a higher glucan content (44). By modulating levels of glucan synthesis, BrpT may therefore control

switching of *S. sanguinis* biofilms from an adherent to a dissemination state. *S. gordonii* also carries a single *gtf* gene, *gtfG*, encoding an enzyme that synthesizes both α -1,3- and α -1,6-linked glucans. This activity was found to promote mixed biofilm formation with *Candida albicans* (45), and a *gtfG* mutant was unable to persist on the tooth surface in rats (46). Expression of GtfG is under the positive regulation of Rgg (47), a homolog of which is present in *S. sanguinis*. As for other oral streptococci, it is likely that expression of Gtfs and thus glucan content of biofilms is a tightly controlled process, affected by multiple factors and transcriptional regulators.

Compared to carbohydrates, the presence of eDNA within EPS is a relatively recent discovery. Nonetheless, growing evidence suggests that eDNA is a critical contributor to cell-to-cell adherence and overall biofilm stability (48). Courtesy of its negative charge, eDNA may facilitate association with the acquired salivary pellicle (49), and cell aggregation was shown to be promoted by eDNA for *S. sanguinis*, although not for *S. gordonii* (50). Visualization of ‘yarn’ and ‘sweater’ structures of eDNA that wrap around cells within *Enterococcus faecalis* biofilms (51) provides compelling evidence for how eDNA may contribute to the structural integrity of biofilms, and similar structures have been seen for biofilms of *S. gordonii* (A.H. Nobbs, unpublished data). Moreover, eDNA regulates the viscoelastic properties of biofilms that allows them to withstand mechanical stress (52), a property that is particularly pertinent to the oral cavity environment. In line with this, the presence of DNABII proteins within EPS of *S. gordonii* and other oral bacterial biofilms was recently found to be essential for eDNA integrity and biofilm structure (53). The regulation of eDNA release is not fully understood, and both lytic and active mechanisms have been reported. For *S. sanguinis* and *S. gordonii*, autolysins LytF

189 and AtIS play significant roles via mechanisms closely linked to H₂O₂ production and
190 competence development. These are discussed in more detail later.

191 Multicellular entities such as biofilms require a high level of coordination, and
192 quorum sensing (QS) circuits and two component signaling systems (TCS) are intimately
193 involved in these processes. One QS system found across several bacteria is LuxS/AI-2.
194 LuxS is an integral component of the activated methyl cycle (AMC) for correct methylation
195 of nucleic acids and proteins, but as a by-product of this cycle generates autoinducer 2
196 (AI-2), an interspecies chemical signal. LuxS mutants in *S. gordonii* and *S. sanguinis* were
197 altered in their ability to form biofilms, and lack of AI-2 resulted in altered *S. gordonii*
198 microcolony architecture (54). However, a definitive role for AI-2 has not been identified
199 and for *S. sanguinis*, a disrupted AMC rather than absence of AI-2 was found to underpin
200 the *luxS* mutant biofilm phenotype (55). A TCS associated with regulation of biofilm
201 formation for both *S. sanguinis* and *S. gordonii* is BfrAB (56). This modulates expression
202 of two ABC transporters (BfrCD, BfrEFG) and a putative membrane-bound
203 metalloprotease (BfrH). Given such functions, it is postulated that this system transports
204 and processes proteins or peptides across the cell membrane that promote biofilm
205 development, although the precise targets have yet to be identified. More recently, defects
206 in TCS SptRS have been found to promote biofilm formation by *S. sanguinis*, associated
207 with elevated levels of H₂O₂ and eDNA (57), while standalone ArcR was identified as a
208 key regulator of *S. gordonii* biofilm development, perhaps via modulation of the
209 phosphotransferase system (58).

210 A final aspect of streptococcal biology that is closely associated with biofilm
211 formation is competence development. This is a QS system that controls transformation,
212 i.e. the capacity for bacteria to actively take up exogenous eDNA, and in species such as

S. sanguinis and *S. gordonii*, is regulated via the *comCDE* operon (59, 60). Gene *comC* encodes a precursor molecule that is cleaved and exported via ABC transporter ComAB, releasing the mature competence stimulating peptide (CSP) into the local environment. For *S. gordonii* this is a 19-amino acid peptide, while the CSP of *S. sanguinis* is 17-amino acid residues, thereby minimizing interspecies cross-talk. The CSP is sensed by TCS ComDE. Once the CSP pheromone exceeds a threshold concentration, ComD phosphorylates ComE, which upregulates expression of early competence genes including *comCDE* and *comAB*, establishing a positive feedback loop, and *comX*. ComX then drives expression of the late competence genes required for DNA binding, uptake and recombination. The coordination of competence and biofilm development contributes to the adaptability of bacteria such as *S. sanguinis* to changing environmental conditions via horizontal gene transfer (HGT). This will be explored in more detail later.

S. SANGUINIS IN COMMUNITY DEVELOPMENT

S. sanguinis and *S. gordonii* can form monospecies biofilms, but within the host the biofilm communities are typically polymicrobial in nature. As pioneer colonizers, these streptococci can have profound consequences for niche occupation and subsequent colonization by incoming species and thus significantly influence whether a community is predisposed to health or disease. Such interactions do not occur at random, but rather are directed in an ordered and temporal manner as a consequence of direct physical engagement (coadhesion), metabolic relationships and interspecies communication.

Alongside streptococci, *Actinomyces* species constitute the predominant, health-associated early colonizers of the oral cavity, and both *S. sanguinis* and *S. gordonii* are able to coadhere with *Actinomyces oris*. This is mediated by recognition of streptococcal

receptor polysaccharide (RPS) containing linkages GalNAc β 1-3Gal or Gal β 1-3GalNAc by the FimA subunit of *A. oris* type 2 fimbriae (61, 62), with variations in the genetic loci for synthesis of RPS (*rps*) and RPS precursors (*rml*, *galE1*, *galE2*) subtly altering the coadhesion profile with different streptococcal species (63). In addition, *S. gordonii* antigen I/II family protein SspB targets an extracellular polysaccharide produced by *A. oris*, although the precise composition and structure of this polysaccharide has yet to be determined (64). Once bound, *A. oris* may then promote *S. gordonii* survival under low arginine conditions by stabilization of arginine biosynthesis (65). Similarly, *S. gordonii* adhesin Hsa binds surface receptor Hag1 of early colonizer *Veillonella* species (66), but this interaction is also underpinned by a strong metabolic dependency. Lacking a fully functional glycolytic pathway, veillonellae must utilize hydroxyl acids for growth. These are provided by streptococci as excreted metabolic waste product lactate and utilization of lactate, in turn, protects streptococci from low pH (67). Once established, this community of pioneer colonizers then supports the incorporation of secondary or late colonizers, with *Fusobacterium nucleatum* serving as an important ‘bridging’ organism due to its promiscuous coadhesion capabilities. *S. sanguinis* supports this engagement via interaction with the arginine-inhibitable adhesin RadD of *F. nucleatum* (68), while a second fusobacterial outer membrane protein, coaggregation mediating protein A (CmpA), has recently been shown to promote biofilm formation with *S. gordonii* (69).

These community interactions with compatible species illustrate how *sanguinis*-group bacteria are able to promote development of a health-associated microbiota. Nonetheless, the social life of these bacteria is not exclusively beneficial and other microbial partnerships may facilitate a more disease-prone state. Such examples have largely been described for *S. gordonii*, rather than for *S. sanguinis*, leading to the

designation of *S. gordonii* as an ‘accessory pathogen’. Some of the best characterized interactions are with periodontopathogens *Porphyromonas gingivalis* (70, 71) and *Aggregatibacter actinomycetemcomitans* (72), and with fungal pathogen *C. albicans* (73). Evidence from in vitro studies and animal models shows that these relationships can enhance both the persistence and virulence potential of the microbes involved (74).

Chemical communication in modulation of the oral microbial community is exemplified by the complex effects of interspecies signaling molecule AI-2. This molecule promotes dual species biofilm formation between *S. gordonii* and *S. oralis* but can also modulate the relative proportions of these species in a concentration-dependent manner (75). Likewise, while AI-2 from *F. nucleatum* promotes biofilm development with *S. gordonii*, it has the opposing effect on *S. oralis* (76). AI-2 from *S. gordonii* is essential for mutualistic biofilm growth with *A. oris* (77), but may also promote biofilm formation with *P. gingivalis* (78) and *C. albicans* (79). Ultimately, QS molecules and peptide pheromones work together with the molecular mechanisms described above to exquisitely coordinate biofilm development. The result is a community optimized to survive and persist under the prevailing environmental conditions in a manner that exceeds the capabilities of the individual component species.

COMPETITIVE BEHAVIOR - INHIBITION BY *S. SANGUINIS* H₂O₂ AND BACTERIOCIN PRODUCTION

Addressed above are examples of synergistic interactions with *sanguinis*-group streptococci that promote incorporation and retention of the partner microbes within the biofilm community. Nonetheless, within a defined ecological niche, resources are limited and thus, competitive forces also work to shape the developing biofilm. One of the best

investigated competitive measures of *S. sanguinis* (and the vast majority of oral streptococci) is the production of H₂O₂ (80). The enzyme responsible for the production of H₂O₂ is pyruvate oxidase, SpxB (81, 82). SpxB is encoded by the majority of commensal oral streptococci, with an unusually high degree of conservation of over 96% amino acid identity when compared to SpxB of *S. sanguinis* strain SK36. SpxB catalyzes the conversion of pyruvate to acetyl phosphate, CO₂, H₂O₂, and ATP. Therefore, besides generating inhibitory amounts of H₂O₂, SpxB confers a growth advantage on the producer via ATP generation during biofilm development (81, 82).

The overall importance of H₂O₂ production is further indicated by the fact that neither *S. sanguinis* nor *spxB*-encoding oral streptococci seem to encode for the H₂O₂ detoxifying enzyme catalase. Consequently, these bacteria are able to produce considerable amounts of H₂O₂ that can influence the surrounding environment and inhibit susceptible species. H₂O₂-dependent competitive behavior faces two challenges. First, SpxB requires oxygen for its activity (83, 84). Its production therefore declines once biofilm formation reaches a certain density resulting in an anaerobic environment. Second, released H₂O₂ is a substrate for detoxifying enzymes like salivary lacto-peroxidase (85). Therefore, the effect of H₂O₂ production on biofilm development is confined to the immediate vicinity of the producer and most likely does not result in active killing of competitors. Rather, H₂O₂ will affect susceptible species just enough to gain a growth advantage. For *S. sanguinis*, the oxygen dependent production of H₂O₂ makes perfect sense from an ecological point of view. As initial colonizer, *S. sanguinis* finds a sparse inhabited environment with enough salivary oxygen tension to promote H₂O₂ production (86). However, once biofilm growth through proliferation and integration of compatible species is sufficient to result in a decline in oxygen tension, *S. sanguinis* will already be

an established member of the community and so H₂O₂ production is no longer required (80). The inhibitory spectrum of H₂O₂ as evaluated *in vitro* with deferred antagonism plate assays includes cariogenic *S. mutans*, as well as periodontopathogens *A. actinomycetemcomitans*, *Prevotella intermedia* and *P. gingivalis* (83, 87).

The genetic regulation of *spxB* expression holds a mystery. A clear repressive function of the carbon catabolite repressor CcpA has been shown since deletion of CcpA in *S. sanguinis* strain SK36 lifts *spxB* repression (88). Further, two *cre* sites (carbon responsive element) for the binding of CcpA have been verified experimentally (89), yet no glucose effect on *spxB* expression and H₂O₂ production can be observed. This is in contrast to other species like *S. gordonii*, which follows classic carbon catabolite repression in the presence of glucose (90). Why CcpA in *S. sanguinis* does not respond to the presence of glucose, and if any environmental factor influences *spxB* expression, has yet to be determined. Compared to *S. gordonii* and other oral streptococci, *S. sanguinis* H₂O₂ production in general seems to be less (89, 91) and a clue to the consequences of low H₂O₂ production and tight control by CcpA might be explained by the increased susceptibility of a *S. sanguinis* CcpA knockout mutant towards its own H₂O₂ production (88). However, whether or not this is a direct effect of H₂O₂ or the result of altered gene expression from other CcpA-controlled genes is not currently known. From the ecological standpoint, the numerical abundance of *S. sanguinis* over other species might not require high production of H₂O₂ while other species like *S. gordonii*, known to be a less prominent member of the oral biofilm, might require more aggressive H₂O₂ production to establish itself within the oral community. Taking into account that oral streptococci intermingle during biofilm formation and can be found at the same location (92), the sum of H₂O₂ production could be more important than the quantity produced by

individual species. Nevertheless, competitive H₂O₂ production and its role in community development is important and can be used to study biofilm dynamics.

Interestingly, *S. sanguinis* is also able to produce antimicrobial activity via bacteriocins. Several reports describe the antimicrobial activity of sanguicins (93, 94). Initial characterization showed bacteriostatic activity against a number of oral species, including *A. naeslundii*, but not periodontopathogens *P. gingivalis*, *P. intermedia* and *F. nucleatum* (53). However, a more recent study using purified sanguicin demonstrated antimicrobial activity against such pathogens (93). Since both studies used a different strain of *S. sanguinis* and reported different molecular weights (65 kDa vs 280 kDa) for the purified peptide, it is most likely that different sanguicins were characterized. It is also worth mentioning that none of the studies determining the effect of H₂O₂ on periodontopathogens or *S. mutans* reported an inhibitory effect when catalase was added to the deferred antagonism assay (87, 95). Therefore, either strain-specific antagonistic activity exists, or the culture conditions used in the studies were selective for specific production of bacteriocins or H₂O₂.

S. sanguinis bacteriocins are also reported to exhibit anti-fungal activity (96, 97). As part of the normal microbiota, *C. albicans* is commonly isolated from subjects, but can also cause problems like oral candidiasis (98). *S. sanguinis* produces a bacteriocin that can cause changes in cell surface hydrophobicity of several *Candida spp.*, a factor that influences the initial adhesion of *Candida* to oral epithelium. Furthermore, the bacteriocin can impair fungal cell membrane permeability and general cell structure (96, 97). How this ultimately affects survival of the fungal cell *in vivo* is not known but, analogous to the effect of H₂O₂ production, it might provide a competitive advantage to *S. sanguinis* by impairing growth of competing oral fungi.

HORIZONTAL GENE TRANSFER AND ANTIBIOTIC RESISTANCE RESERVOIR

An important feature of oral streptococci is the ability to take up eDNA when developing competence, as discussed earlier. While the process of competence development is well characterized at the molecular level (99), the origin of DNA in the environment and the molecular mechanisms of DNA release from bacterial cells are less well understood. As a vital part of the oral biofilm matrix, eDNA available for uptake by competent bacteria is quite abundant (100, 101). In general, cell lysis can contribute to the release of DNA from bacterial cells. This process can be an active autolysis process involving murein hydrolases that weaken or completely lyse the cell wall, consequently expelling DNA into the environment (102). Interestingly, both *S. sanguinis* and *S. gordonii* release DNA in a H₂O₂-dependent manner under aerobic conditions (83). The eDNA is largely intact, high molecular weight DNA of chromosomal origin. Release of eDNA peaks when *S. sanguinis* grows aerobically under optimal H₂O₂-producing conditions and release declines when cells are grown under oxygen-limiting conditions, when no H₂O₂ is produced. Furthermore, deletion of *spxB* severely impacts the release (83). However, eDNA release can be induced even under anaerobic conditions with the addition of H₂O₂, as shown for *S. gordonii*, but only when cells are metabolically active (103). Addition of H₂O₂ to cells suspended in buffer will not release DNA, indicating the requirement for active metabolism, gene expression or protein synthesis. This is also consistent with the observation of a time delay between the production of H₂O₂ and the appearance of the released DNA, and the evidence that addition of chloramphenicol, known to block synthesis of new proteins, also blocks release of DNA (103). This is relevant in the context of the oral biofilm, where SpxB-positive streptococci that have access to oxygen can

produce H₂O₂ that might then diffuse through the oral biofilm with limited or no oxygen. Remarkably, the H₂O₂-dependent release of DNA is not the result of obvious cell lysis (50), as reported for other streptococci like *S. pneumoniae* (104). Although the exact molecular mechanism involved in the release is not known at this moment, *S. sanguinis* might encode a dedicated system for eDNA release. This could involve partial lysis and/or active transport across the membrane. In a recent study, the transport of eDNA in *H. influenzae* was found to be dependent upon competence-related protein ComE (105), the transporter involved in the uptake of DNA. *S. sanguinis* encodes for ComE and intriguingly expression of competence genes seems to be increased under aerobic conditions ideal for H₂O₂ production in streptococci (83, 103). However, experimental evidence that this mechanism is conserved in *S. sanguinis* is not available. When grown under anaerobic conditions, eDNA release is not completely absent suggesting another mechanism involved in the release (50). One autolytic enzyme involved in this process in *S. sanguinis* is LytF, also under the control of the competence system as one of the early competence genes (106). LytF is a secreted autolysin and can be recovered from the environment in its active form. LytF is a fratricin, but also acts on neighboring cells causing release of eDNA not only from *S. sanguinis* but also other streptococci (106). In general, streptococci seem to control or connect competence development with the release of eDNA (107), exemplifying their role as masters of efficacy. Utilizing this approach increases the chance that DNA is available for uptake and transformation.

Uptake of eDNA has two pronounced functions; first, it serves in horizontal gene transfer during bacterial transformation and therefore plays a pivotal role in the promotion of diversity among oral bacterial species, and is an important mechanism of evolution allowing the acquisition of new genetic traits stored in the genomic information contained

in the multispecies biofilm (108). Second, eDNA in the oral cavity can serve as a reservoir for diverse antibiotic resistance mechanisms (109). A recent comparison of *S. sanguinis* and *S. gordonii* genomes revealed the presence of several genes potentially associated with antibiotic resistance determinants, including GNAT acetyltransferases, *parE*, and TetR family regulators (110). Combined they potentially provide resistance to diverse antibiotics like aminoglycosides, fluoroquinolones and many more. Additionally, several genes were identified encoding anion channels in *S. sanguinis* that confer resistance to fluoride (111), particularly important since fluoride is successfully used to prevent caries. Although the direct transfer of antibiotic resistance determinants via eDNA from other species to *S. sanguinis* has not been confirmed, evidence exists that in general the acquisition of new genetic traits occurs. For example, the pathway for vitamin B₁₂ biosynthesis as well as the degradation of ethanolamine and propanediol, which are encoded on a large genomic region, has been acquired through horizontal gene transfer (112), although it is unknown if this was mediated through conjugation, transduction or transformation. The best evidence for the potential of H₂O₂-induced transfer of antibiotic resistance was shown with *S. gordonii*, using engineered strains carrying antibiotic resistance cassettes. Co-incubation of strains encoding distinct antibiotic cassettes under ideal H₂O₂-producing conditions increased the occurrence of intraspecies genetic exchange by 300-fold when compared to non-permissive conditions (103). Overall, antibiotic resistance in the oral biofilm seems to be mainly acquired through horizontal gene transfer as suggested by a recent review (113). The dental biofilm is an ideal environment for the development and transfer of antibiotic resistance, even under conditions where no external pressure through antibiotic administration is present. This is supported by the finding that biofilm evolution and selective pressure through competitive

bacterial interactions mediated by bacteriocins alone gave rise to antibiotic resistance (114). The oral microbiome is able to elicit this evolutionary pressure due to the abundance of bacteriocins (115).

S. SANGUINIS AS A MODULATOR OF THE HOST

While the predominant ecological niche of *S. sanguinis* is the tooth surface, dental plaque formed at the gingival margin brings the oral biofilm into contact with the oral epithelium and associated immune defenses. The host-microbe interplay that occurs at these sites represents a critical step in determining progression of the biofilm community below the gum line, and potential transition from oral health to onset of gingivitis, periodontitis or other disease manifestations. Another facet of the *S. sanguinis* persona as coordinator of the microbial community is therefore its interactions with host tissues.

Under conditions of oral health, an equilibrium exists between microbiota and host, representing a delicate balance of antimicrobial factors from immune cells, together with pro- and anti-inflammatory molecules released from the host in response to the sustained microbial challenge. By contrast, disruption of this homeostasis is the hallmark of chronic inflammatory periodontal disease and resultant tissue damage. In keeping with this, *S. sanguinis* biofilms were found to be a poor stimulant of proinflammatory cytokines IL-1 α , IL-6 and IL-8 from OKF4 oral epithelial cells compared to biofilms of *F. nucleatum* (116). Likewise, in contrast to cell wall extracts of *F. nucleatum* or *P. gingivalis*, those of *S. sanguinis* failed to induce significant upregulation by gingival keratinocytes of genes encoding human β defensin peptides, proinflammatory cytokines (e.g. IL-8) or matrix metalloproteinase-9. These keratinocyte responses were mediated by TLR2, with

differences in acylation patterns of bacterial lipopeptides purported to underpin the differential outcomes of TLR2 stimulation by the bacterial species (117). Moreover, while only a weak stimulant of host responses itself, *S. sanguinis* is also able to suppress the effects of other microbes. In mixed culture, *S. sanguinis* impaired induction of IL-8 release from gingival HOK-18A epithelial cells by *A. actinomycetemcomitans*. Such effects were also seen with *S. sanguinis* spent culture medium alone, implying a secreted molecule as the mediator (118). Similarly, through blocking LPS engagement with monocyte receptors LPS-binding protein (LBP) and CD14, peptidoglycan from *S. sanguinis* was able to inhibit induction of genes encoding TNF α , IL-6 and IL-8 by periodontopathogens *P. gingivalis*, *A. actinomycetemcomitans* and *Tannerella forsythia* (119). This mechanism correlates well with the observation that Gram-positive bacteria release large quantities of peptidoglycan fragments, muropeptides, during cell division (120). Such coordination of both host responses and of the oral microbiota is likely critical to the role of *S. sanguinis* as a health-associated member of the oral biofilm community.

S. SANGUINIS AS AN ETIOLOGICAL AGENT OF EXTRAORAL DISEASES

Despite its main role as a benign oral commensal, the name *S. sanguinis* derives from its role in cardiovascular disease infective endocarditis (IE). In a note to the Journal of Bacteriology, Niven and White described a new species isolated from approximately 100 cases of subacute bacterial endocarditis (121). About one third of the isolates failed characterization as previously described streptococci and were referred to as *Streptococcus s.b.e.* (for subacute bacterial endocarditis) (121). Overall the group was quite homogeneous in its physiological characteristics (122). Further serological

characterization confirmed the isolation of a new species since no cross reactivity was observed between rabbit serum from *Streptococcus s.b.e.* and other identified streptococci belonging to various Lancefield groups (123). Interestingly, isolation of *Streptococcus s.b.e.* from the human throat was unsuccessful at that time, despite testing over 800 streptococcal isolates. The only other positive culture came from an extracted tooth, which we now know coincides with the preferred colonization site. However, in the original publication, the natural habitat was not identified and the only source was from the blood of endocarditis patients, hence the species name *sanguis*; Latin for blood (122, 124). This has been changed fairly recently to the grammatically correct version, *S. sanguinis* (125).

IE is a relatively rare, but potentially fatal disease and can affect the heart valves or endocardium. The annual incidence ranges from 3-7 cases per 100,000 people per year and has been relatively constant, whereas the etiology of IE has changed over time (126, 127). A major concern with this disease is that mortality rates remain high; the in-hospital mortality rate ranges from 15-22%, with a 5-year mortality rate around 40%. Currently, *Staphylococcus aureus* is the most common pathogen associated with IE. However, oral streptococci are responsible for an estimated 35-40% of cases, with *sanguinis*-group species the most common isolates (128).

Understanding the molecular mechanisms that might contribute to the capacity for *S. sanguinis* to cause IE has benefited from the availability of an excellent animal model for IE. New Zealand white rabbits can be catheterized at specific sides of the heart causing injury-induced IE after bacterial blood inoculation (129). The injury provides exposed ECM components, fibrin and platelets for attachment of the bacterial cells and subsequent aberrant clot formation, which leads to infective vegetations forming on the heart valves.

Todd Kitten's group, Virginia Commonwealth University, have used this model to decipher molecular determinants expressed by *S. sanguinis* during the infection process. Using a random signature-tagged mutagenesis approach, six chromosomal loci were identified from an initial screen of 800 mutants. The mutants carried transposons in an intergenic region and in genes encoding undecaprenol kinase, homoserine kinase, anaerobic ribonucleotide reductase, adenylosuccinate lyase, and a hypothetical protein (130). The screening method is certainly elegant but has some technical limitations, since important determinants like surface-exposed proteins, which have been shown to contribute to the infection process (131), were missed. Nonetheless, the identification of determinants regarded as house-keeping genes involved in cell wall (undecaprenol kinase), amino acid (homoserine kinase) and nucleic acid (ribonucleotide reductase) synthesis suggested a potential role for these genes in pathogenesis that had not previously been appreciated. Furthermore, since these genes are not found in humans, they may represent potential targets for drug development. However, a functional analysis of the clonal structure of *S. sanguinis* strains isolated from the oral cavity and from subjects with IE demonstrated that house-keeping and virulence genes are subject to considerable intra-species recombination events. Thus potential drug targets in such genes may correlate with a high potential for the selection of resistant mutants (132). Interestingly, the same study also concluded that the endocarditis strains did not form a distinct sub-cluster. This supports the notion that *S. sanguinis* strains are human pathobionts, and thus all strains have the potential to cause IE (132).

Following on from this study, a targeted signature-tagged mutagenesis approach was employed to identify surface proteins important in IE. Thirty three proteins were classified as cell wall-associated and of these, mutants in 31 were tested in the

aforementioned rabbit model of IE, alongside mutants in 3 sortase genes (133). Interestingly, no single cell wall-associated protein was found to be essential for the development of IE (133). It may be concluded from this that no specific virulence determinants are required by *S. sanguinis* to cause IE. Rather, what is expressed and present during colonization of the oral cavity may be sufficient to colonize the endocardium. In support of this, surface adhesins Hsa, PadA and SspA/B from *S. gordonii* have been implicated in IE through their engagement with platelets, yet also contribute to the capacity for *S. gordonii* to bind salivary pellicle and other oral microbes (134-137). Such highly optimized genomes might explain the success of these bacteria as early colonizers, with their ability to cause IE on rare occasions coincident in their ubiquitous presence and abundance within the oral cavity.

Mutations in the genes encoding lipoprotein SsaB (a putative manganese transport protein) (38), *spxA1* (encoding a global regulator involved in H₂O₂ production) (138) and *nox* (encoding an NADH oxidase that also influences H₂O₂ production) (139), together with the anaerobic ribonucleotide reductase already mentioned, have all been shown to impair the capacity of *S. sanguinis* to cause IE. This suggests that the ability of *S. sanguinis* to adapt to differences in oxygen tension and the production of and resistance to reactive oxygen species are important in the development of IE, although molecular details have yet to be explained. A surface-bound nuclease, designated SWAN (streptococcal wall-anchored nuclease), has also been proposed to enable *S. sanguinis* to evade killing by neutrophil extracellular traps (NETs), and so promote survival both in the bloodstream and within infective vegetations (140).

Survival within the bloodstream to access extraoral sites and induction of thrombosis are key steps in IE, but these capabilities also associate *S. sanguinis* with

other systemic diseases. These include meningitis, following infection of the lining of the spinal cord or brain, and disseminated intravascular coagulation, in which aberrant activation of the coagulation cascade leads to the formation of small clots that can occlude blood flow to major organs and tissues (141, 142). It is also widely recognized that oral streptococci such as *S. sanguinis* exist in biofilm communities with *Pseudomonas aeruginosa* in the lungs of cystic fibrosis (CF) patients. However, with evidence that H₂O₂ production by these streptococci can impair *P. aeruginosa* growth (143), the impact of *S. sanguinis* on CF disease progression remains to be fully understood.

CONCLUSION/OUTLOOK

With the impact upon the individual, it is easy to understand why disease outcomes have historically dominated microbiological research. Nonetheless, technological advances are providing a growing appreciation for the importance of our resident microbiota. Within the oral cavity, bacteria such as *S. sanguinis* are ubiquitous and abundant, reflecting their evolution to be exquisitely adapted to colonization of their ecological niche. As a consequence, *S. sanguinis* is able to orchestrate accretion of the dental plaque biofilm, promoting the acquisition of beneficial microbes while serving as an imposing competitor to others, and ultimately serving as the foundation of a health-associated biofilm community. Complex molecular mechanisms regulating physical interactions and communication networks underpin these capabilities. If these can be understood at both the molecular and ecological level, they offer immense potential for exploitation in the development of novel strategies to combat infections from a point of health as opposed to disease.

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FIGURE LEGENDS

Figure 1. Summary of key *Streptococcus sanguinis* components important in commensalism. The schematic shows important components for the role of *S. sanguinis* as a commensal organism, including community integration and biofilm development, community interference and streptococcal antagonism, and interactions with salivary proteins, host cells, and the immune system. Pg, *Porphyromonas gingivalis*; Fn, *Fusobacterium nucleatum*; Sg, *Streptococcus gordonii*; eDNA, extracellular DNA; CSP, competence stimulating peptide. Reprinted with permission from Kreth et al. (2017). The road less traveled – defining molecular commensalism with *Streptococcus sanguinis*. *Mol Oral Microbiol* 32:181–196. doi:10.1111/omi.12170.